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<p>(54) Title: TARGETING AND PROLONGING ASSOCIATION OF DRUGS TO THE LUMINAL SURFACE OF THE PULMONARY VASCULAR ENDOTHELIAL CELLS  (57) Abstract  Methods for targeting and prolonging association of a selected drug to the luminal surface of pulmonary vascular endothelium of an animal are provided wherein a selected drug is administered to an animal in combination with a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vasculature. This method is particularly useful in dissolution of fibrin clots or prevention of the intravascular coagulation in the pulmonary vasculature.</p>		

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TARGETING AND PROLONGING ASSOCIATION OF DRUGS TO THE  
LUMINAL SURFACE OF THE PULMONARY VASCULAR ENDOTHELIAL CELLS

Background of the Invention

Pulmonary vasculature is anatomically predisposed to  
5 deposition of fibrin and thromboemboli formed in the  
vasculature (for example, upon deep vein thrombosis). Both  
emboli and fibrin lodged in the lung play an important role  
in the pulmonary and cardiovascular pathology and contribute  
significantly to morbidity and mortality of disease conditions  
10 including, but not limited to, thrombosis, atherosclerosis,  
deep vein thrombosis, diabetes, adult respiratory distress  
syndrome, pulmonary embolism, shock and sepsis.  
Anticoagulants (e.g., heparin) are useful in preventing  
formation of intravascular fibrin clots, whereas fibrinolytics  
15 (e.g., plasminogen activators) are useful for dissolution of  
fibrin clots. Both anticoagulants and fibrinolytics, however,  
undergo inactivation and elimination from the bloodstream.  
This restricts their applicability for treatment of pulmonary  
embolism. Administration of large doses and/or multiple  
20 injections of a drug to compensate for  
elimination/inactivation impose inconvenience in treatment and  
high risk of harmful side effects. Uncontrolled bleeding is  
an example of such side effects of prolonged administration  
or a large dose of anticoagulants or fibrinolytics.  
25 Augmentation of anticoagulant or/and fibrinolytic  
potential of the luminal surface of endothelial cells lining

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pulmonary vessels thus represents an important therapeutic strategy for treatment or/and prevention of disease conditions associated with or manifested by pulmonary embolism and fibrin deposition. Because these therapeutics must have access to the blood components in order to control coagulation or activate fibrinolysis, a requirement for such a strategy is that the anticoagulant or fibrinolytic agent be associated for a prolonged time with the luminal surface of the pulmonary endothelium.

One approach to attain this objective is to conjugate a drug to an antibody against surface endothelial molecules. This conjugation provides selective delivery, also referred to herein as targeting, of a drug to endothelium and prolonged association of a drug with endothelium. Therapeutic enzymes and genetic material conjugated to such antibodies have been demonstrated to bind to the endothelial cells *in vitro* and *in vivo* after injection in animals. Since the lungs contain approximately 30% of the total amount of endothelial cells in the body and receive a whole cardiac output of venous blood, antibodies against endothelial antigens tend to accumulate in the lung after intravenous injection. For example, Kennel et al. have described an antibody against thrombomodulin which recognizes endothelial surface *in vivo*, accumulates in the pulmonary vasculature and is capable of delivery of conjugated liposomes to the pulmonary endothelium (Kennel et al. 1990 *Nucl. Med. Biol.* 17:193-100; Trubetskoy et al. 1992 *Biochim. Biophys. Acta* 1131:311-313). An antibody against angiotensin-converting enzyme (ACE) has been described which possesses very similar properties (Danilov et al. 1991 *Lab. Invest.* 64:118-124). Therapeutic enzymes such as catalase, superoxide dismutase and plasminogen activators conjugated with ACE antibody have been demonstrated to accumulate in the lungs after intravascular injection (Muzykantov et al. 1996 *Proc. Nat'l Acad. Sci. USA* 93:5213-5218; Muzykantov et al.

1997 *J. Pharm. Exp. Therap.* 279:1026-1034). In addition, an antibody against E-selectin has been described which binds to and delivers liposomes to the cytokine-activated endothelium in cell culture (Spragg et al. 1997 *Proc. Nat'l Acad. Sci. USA* 5 94:8795-8800). A PECAM antibody conjugated with streptavidin has also been recently described which provides an effective carrier for delivery of drugs to the endothelium (Muzykantov et al. 1998 *Am. J. Resp. Crit. Care Med.* 157:A203).

However, endothelial cells internalize antibodies  
10 against thrombomodulin (Muzykantov et al. 1997 *Circulation* 96:I43-44), ACE (Muzykantov et al. 1996 *Am. J. Physiol.* 270:L704-713), E-selectin (Spragg et al. 1997 *Proc. Nat'l Acad. Sci. USA* 94:8795-8800) and anti-PECAM/streptavidin complex (Muzykantov et al. 1998 *Am. J. Resp. Crit. Care*  
15 *Medicine* 157:A203). Thus, while these carriers provide intracellular delivery, a feature which may be useful for targeting of genes and some other therapeutic agents, anticoagulants or fibrinolytics must escape internalization and remain on the luminal surface in order to control blood  
20 components. Accordingly, these carrier antibodies are of limited use in the delivery of anticoagulants, fibrinolytics or other drugs wherein their therapeutic action is localized to the blood.

An ICAM-1 monoclonal antibody, mAb 1A29 has also been  
25 described which accumulates in rat lungs following i.v. injection. Conjugation of catalase to this ICAM-1 monoclonal antibody via a streptavidin-biotin crosslinker resulted in accumulation of the anti-ICAM-1 conjugated catalase in the lung and protection of the lung from damage by extracellular  
30 oxidants (Muzykantov et al. *Am. J. Resp. Crit. Care Medicine* 1997 155(4):p.A187). Radiolabeled mAb 1A29 has also been shown to accumulate in the vasculature challenged with pro-inflammatory agents TNF and endotoxin (Mulligan et al. 1993 *Am. J. Pathol.* 142:1739-1749). In addition, this

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antibody has been shown to react with normal endothelial cells in the rat vasculature and that injection of TNF or endotoxin stimulates endothelial binding of mAb 1A29 (Panes et al. 1995 *Am. J. Physiol.* 269:H1955-1964). This antibody has also been  
5 shown to attenuate vascular injury induced by activated leukocytes via blocking of their adhesion to the endothelial cells.

It has now been found that monoclonal antibodies against the endothelial surface antigen ICAM-1 bind effectively to the  
10 endothelial cells without subsequent internalization. Conjugation of a drug to a non-internalizable antibody such as the ICAM-1 monoclonal antibody which binds to an antigen on the luminal surface of the pulmonary vasculature provides a useful means for targeted delivery and retention of the drug  
15 on the luminal surface, or blood compartment, of the pulmonary vasculature.

#### Summary of the Invention

An object of the present invention is to provide a method for targeting and prolonging association of drugs, the  
20 therapeutic action of which must be localized in the blood compartment of the pulmonary vasculature, to the luminal surface of either normal or inflammation-affected pulmonary vascular endothelium, which comprises utilization of a non-internalizable antibody which binds to an antigen on the  
25 luminal surface of the pulmonary vasculature, for example an anti-ICAM-1 antibody such as anti-ICAM-1 mAb 1A29, as an affinity carrier or a membrane anchor for the targeting and retention of the drugs on the luminal surface of the endothelium.

30 Another object of the present invention is to provide a method of administration of a drug, the therapeutic action of which must be localized in the blood compartment of the pulmonary vasculature which comprises either: 1) conjugation of a selected drug with a non-internalizable antibody which

binds to an antigen on the luminal surface of the pulmonary vasculature, for example an anti-ICAM-1 antibody such as anti-ICAM-1 mAb 1A29, leading to formation of a "non-internalizable antibody/drug" complex and systemic  
5 administration of the said complex to an animal; or ii) step-wise systemic administration to an animal of a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vasculature followed by systemic administration of a drug chemically modified in the  
10 way that allows the drug to recognize and bind to the non-internalizable antibody bound to the luminal surface to avoid internalization.

Another object of the present invention is to provide a method for dissolution of fibrin clots or attenuation of the  
15 intravascular coagulation in the lung of an animal which comprises systemically administering to the animal a fibrinolytic or anticoagulant agent in combination with a non-internalizable monoclonal antibody which binds to an antigen on the luminal surface of the pulmonary vasculature.

## 20 Detailed Description of the Invention

ICAM-1 (InterCellular Adhesion Molecule-1) is a transmembrane protein anchored in the plasma membrane of several cell types, including endothelial cells. ICAM-1 is present on the surface of normal (non-stimulated) endothelium.  
25 Inflammatory agents cause elevation of ICAM-1 levels on the endothelial surface. Thus, inflammation-engaged endothelium possesses even more binding sites for ICAM-1 antibody than normal endothelium.

Monoclonal antibodies against ICAM-1 have been  
30 demonstrated to be useful as carriers of agents to the pulmonary endothelium. Accordingly, studies were performed to ascertain the usefulness of these anti-ICAM-1 antibodies in delivery of drugs such as fibrinolytics and anticoagulants. Anti-ICAM-1 mAb 1A29, a monoclonal antibody serving as an

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example in the present invention which is a mouse IgG1 class monoclonal antibody reacting with rat ICAM-1, was used in these studies. This antibody is commercially available from a number of vendors including PharMingen (San Diego, CA),  
5 Endogen, Inc. (Boston, MA) and Serotec Ltd (United Kingdom). Since lack of internalization is an obligatory for the therapeutic action of fibrinolytics and anticoagulants, studies were performed to determine how endothelial cells in cell culture or in the lung blood vessels internalize  
10 radiolabeled mAb 1A29. These experiments demonstrated that unlike antibodies to other endothelial antigens, endothelial cells internalize anti-ICAM-1 extremely poorly.

For example, in cell culture, internalization of  $^{125}\text{I}$ -mAb 1A29 did not exceed 5-10%. In contrast,  $^{125}\text{I}$ -mAb against other  
15 endothelial antigens such as thrombomodulin and ACE displayed 60-80% internalization.

In addition, pulmonary uptake of anti-ICAM-1 is independent of the temperature thus indicating that this antibody is not internalized. Uptake of  $^{125}\text{I}$ -mAb 1A29 in the  
20 isolated perfused rat lung was  $18.7 \pm 3.2\%$  at  $37^\circ\text{C}$  and  $18.1 \pm 3.3\%$  at  $4^\circ\text{C}$ . In contrast, pulmonary uptake of  $^{125}\text{I}$ -mAb against ACE was twice as low at  $4^\circ\text{C}$  as compared to  $37^\circ\text{C}$  (Muzykantov et al. 1996 *Am. J. Physiol.* 270:L704-713).

Further, experiments conducted to evaluate whether mAb  
25 1A29 associated with the endothelial cells disappears from the luminal surface in the lung showed that in sharp contrast to anti-ACE and other known carrier antibodies, anti-ICAM-1 is bound to the external surface of the pulmonary endothelial cells for a prolonged time and does not disappear from the  
30 lumen. In these experiments, rat lungs were perfused with biotinylated mAb 1A29 (b-mAb 1A29) and after elimination of non-bound antibody consequently perfused  $^{125}\text{I}$ -streptavidin in the lungs. Pulmonary uptake of  $^{125}\text{I}$ -streptavidin was at the same level when streptavidin was added to the perfusion either  
35 5 or 60 minutes after elimination of b-mAb 1A29. In contrast,



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it has been shown that consequent uptake of  $^{125}\text{I}$ -streptavidin decreases dramatically within an hour after elimination of the biotinylated anti-ACE from the perfusate, thus indicating that b-anti-ACE disappears from the luminal surface.

5 Taken together, these results indicate that endothelial cells effectively bind anti-ICAM-1 antibodies such as mAb 1A29, yet do not internalize this carrier. Accordingly, drugs targeted to endothelial cells by anti-ICAM-1 will be exposed to the vascular lumen for a prolonged period of time and, 10 therefore, will be able to more effectively interact with plasma protein thus regulating coagulation and fibrinolysis.

Pulmonary uptake of fibrinolytics, namely,  $^{125}\text{I}$ -tPA and  $^{125}\text{I}$ -streptokinase conjugated with anti-ICAM-1 mAb 1A29, in the perfused rat lungs and after injection *in vivo* in rats was 15 evaluated. As Table 1 shows, antibody-conjugated fibrinolytics, but not control IgG-conjugated enzymes accumulate in the rat lungs in both models, thus indicating that anti-ICAM-1 antibody indeed provides delivery of therapeutics to the luminal surface of the pulmonary vascular 20 endothelium.

**Table 1. Pulmonary uptake of radiolabeled therapeutic enzymes conjugated to either control IgG or to anti-ICAM-1 mAb 1A29.**

	Carrier	Streptokinase	tPA
Perfused lung	IgG	1.3±0.7	1.6±0.4
Perfused lung	anti-ICAM	12.4±1.7	15.3±1.6
25 Lung, <i>in vivo</i>	IgG	ND	0.22±0.1
Lung, <i>in vivo</i>	anti-ICAM	ND	6.1±0.7

Data in Table 1 are presented as % of injected dose per gram of the lung tissue,  $M \pm SD$ ,  $n=3$ . Radioactivity in the lung was determined 1 hour after start of the perfusion or after 30 intravenous injection in intact anesthetized rats.

Further, subsequent perfusion of  $^{125}\text{I}$ -tPA/streptavidin complex 60 minutes after accumulation of biotinylated mAb 1A29 in the lungs provided pulmonary uptake of  $17.5 \pm 2.7\%$  of  $^{125}\text{I}$ -tPA. In a control experiment, in the absence of the first  
5 step of the targeting (i.e., without perfusion of biotinylated mAb 1A29) uptake of  $^{125}\text{I}$ -tPA was equal to  $0.7 \pm 0.2\%$ , thus demonstrating the specificity of the targeting to b-anti-ICAM-1 attached to the pulmonary endothelium. Comparison of the result of step-wise targeting described  
10 above ( $17.5 \pm 2.7\%$ ) with that of direct targeting ( $15.3 \pm 1.6\%$ , see Table 1) provides additional evidence that endothelial cells in the lung do not internalize mAb 1A29 as step-wise targeting would clearly be compromised by disappearance of b-mAb 1A29 from the lumen.

15 The functional activity of tPA targeted to the pulmonary endothelium via an anti-ICAM-1 monoclonal antibody was also evaluated. In these experiments, isolated rat lungs were perfused for 1 hour with  $100 \mu\text{g}$  of mAb 1A29/tPA or IgG/tPA or with buffer. After elimination of non-bound material, lung  
20 tissue homogenates were prepared. Samples of lung homogenates were added to radiolabeled fibrin clot and incubated for 90 minutes at  $37^\circ\text{C}$ . Homogenate obtained from lungs perfused with conjugate-free buffer induced  $6.5 \pm 1.0\%$  fibrinolysis (background level). The homogenate obtained from the lungs  
25 perfused with IgG/tPA complex induced  $9.2 \pm 2.5\%$  fibrinolysis. This value is not significantly different from the background fibrinolysis level. In a sharp contrast, homogenate obtained from the lungs perfused with anti-ICAM-1/tPA complex induced  
30 of tPA to the luminal surface of the pulmonary endothelium markedly enhances fibrinolytic activity of the lung vasculature. Further, immunotargeting of tPA (or other plasminogen activators) will augment local fibrinolytic potential of endothelium in the focus of the pulmonary  
35 vasculature due to local generation of plasmin.

Accordingly, administration of a non-internalizable antibody such as anti-ICAM-1 antibody in combination with a selected drug, the therapeutic action of which must be localized in the blood compartment of the pulmonary vasculature, provides a useful means for targeting and prolonging association of the drug to the luminal surface of either normal or inflammation-affected pulmonary vascular endothelium.

By "non-internalizable antibody" it is meant an antibody which binds to an antigen on the luminal surface of the pulmonary vasculature such as the anti-ICAM-1 antibody, mAb 1A29, which is determined not to be internalized by cultured human endothelial cells as described in Example 2 and/or is shown to be temperature independent in pulmonary uptake experiments in isolated lung perfusions as described in Example 3. Non-internalizable antibodies other than the anti-ICAM-1 antibody described herein which are also useful in the instant invention can thus be identified routinely by those of skill in the art in accordance with teachings provided herein.

By "selected drug" in the present invention, it is meant to include any therapeutic agent, the therapeutic action of which must be localized in the blood compartment of the pulmonary vasculature. Examples include, but are not limited to fibrinolytics including plasminogen activators and anticoagulants.

By "prolonging association", it is meant that the drug when administered in combination with a non-internalizable antibody such as anti-ICAM-1 antibody undergoes slower inactivation and/or elimination from the bloodstream as compared to the same drug administered alone.

By "in combination" it is meant that a selected drug is administered either as a non-internalizable antibody/drug complex or in a stepwise manner wherein the non-internalizable antibody is administered first followed by administration of

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the selected drug. Thus, in one embodiment, a selected drug can be conjugated to a non-internalizable antibody to form a non-internalizable antibody/drug complex by a number of different methods well known to those of skill in the art.

5 For example, conjugation of anti-ICAM-1 to a selected drug such as a plasminogen activator may be performed using a homo-bifunctional cross-linking agent. Such cross-linking agents offer conjugation of two proteins via chemical modification of the same functional groups on both proteins. Since all

10 proteins contain amino groups, this class of cross-linkers usually produces intermolecular complexes by cross-linking of their amino groups (Sakharov et al. 1988 *Thrombosis Res.* 49:481-488). Introduction of disulfide groups in two proteins by incubation with equimolar amounts of N-succinimidyl-3-(2-

15 pyridyldithio)propionate (SPDP) followed by reduction of the disulfide groups on one of the proteins also allows for conjugation of the two proteins (Cavallaro et al. 1993 *J. Biol. Chem.* 268:23186-23190). The hetero-bifunctional cross-linking agent, m-maleimidobenzoic acid N-hydroxysuccinimide

20 ester can also be used for conjugating an SPDP-treated plasminogen activator with any protein including anti-ICAM-1 or, vice versa, SPDP-treated anti-ICAM-1 with a plasminogen activator. A selected drug can also be coupled with the antibody using a bi-functional antibody chimera possessing

25 affinity for both the selected drug and the antibody. In a preferred embodiment, streptavidin-biotin cross-linking is used. In this embodiment, both the antibody and selected drug are modified with biotin ester which allows for further intermolecular conjugation of the biotinylated molecules by

30 streptavidin. Streptavidin-mediated cross-linking of biotinylated proteins is a widely used biochemical method. Further, as demonstrated herein, the enzymatic activity of tPA is not reduced in the course of biotinylation, conjugation with streptavidin and with biotinylated anti-ICAM-1. In

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addition, the ability of the antibody to specifically target the lung is not altered by this process.

In another embodiment, the selected drug is chemically modified to recognize and bind a non-internalizable antibody  
5 such as anti-ICAM-1 antibody associated with or bound to the luminal surface of the endothelium. In this embodiment, referred to herein as step-wise systemic administration, biotinylated non-internalizable antibody is systemically administered to the animal so that the antibody binds to a  
10 specific antigen on the luminal surface of the pulmonary vasculature. The selected chemically modified drug is then systemically administered to the animal so that the selected drug binds to the non-internalizable antibody associated with the luminal surface thereby avoiding internalization. For  
15 example, the plasminogen activator, tPA, has been chemically conjugated with streptavidin, a molecule recognizing a biotinylated anti-ICAM-1 antibody associated with endothelial surface. Thus tPA/streptavidin complex binds to endothelium-bound anti-ICAM antibody.

20 Administration of a selected drug in combination with a non-internalizable antibody is particularly useful in dissolution of fibrin clots or prevention of the intravascular coagulation in the lung. In this embodiment, it is preferred that the selected drug be a fibrinolytic agent, preferably a  
25 plasminogen activator, or an anticoagulant such as chemically modified heparin, hirudin or recombinant thrombomodulin.

By "systemic administration" it is meant to include intravenous, intraarterial injections and infusions, as well as local delivery via a vascular catheter into selected  
30 vascular bed (for example, pulmonary artery).

By "animal" it is meant to include mammals, most preferably humans.

The following nonlimiting examples are provided to further illustrate the present invention.

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**EXAMPLES****Example 1: Biotinylation, radiolabeling of proteins, and preparation of the conjugates**

Biotin ester, 6-biotinylaminocaproic acid N-  
5 hydroxysuccinimide ester (BxNHS) was dissolved in 100% dimethylformamide to a final concentration of 10 mM or 1 mM. Control mouse IgG and anti-ICAM-1 mAb 1A29 were biotinylated at ten-fold molar excess of BxNHS. Eight  $\mu$ l of fresh 1 mM BxNHS were added to 100  $\mu$ l of antibody solution (1 mg/ml in  
10 borate buffered saline, BBS, pH 8.1). After a 1 hour incubation on ice, an excess of non-reacted BxNHS was eliminated by overnight dialysis. Streptokinase and tPA were biotinylated by the same reagent at 10-fold molar excess of BxNHS, as described above. Biotinylated antibodies, b-  
15 streptokinase, b-tPA or streptavidin were radiolabeled with  $^{125}$ Iodine using Iodogen-coated tubes according to the manufacturer's recommendations (Pierce). Incubation of 100  $\mu$ g of a biotinylated protein and 100  $\mu$ Ci of Sodium  $^{125}$ Iodide in a tube coated with 100  $\mu$ g of Iodogen for 20 minutes on ice  
20 yields streptavidin with a specific radioactivity of approximately 500 cpm per ng. An excess of iodine was eliminated by dialysis. More than 95% of radiolabeled proteins were precipitable by TCA.

Tri-molecular heteropolymer complexes b-tPA/SA/b-IgG,  
25 b-tPA/SA/b-anti-ICAM-1, b-streptokinase/SA/b-IgG and b-streptokinase/SA/b-anti-ICAM-1 were prepared by a two-step procedure. Specifically, at the first step, streptavidin (SA) and b-tPA were mixed at a molar ratio equal to 2, in order to form bi-molecular complexes b-tPA/SA. Accordingly, 10  $\mu$ l of  
30 BBS containing 10  $\mu$ g of radiolabeled b-tPA was mixed with 10  $\mu$ l of BBS containing 20  $\mu$ g of streptavidin and incubated for 1 hour on ice. The mixture was then divided into two 10  $\mu$ l portions. To the first portion was added 15  $\mu$ l of BBS containing 20  $\mu$ g of biotinylated anti-ICAM-1. To the second  
35 portion was added 15  $\mu$ l of BBS containing 20  $\mu$ g of control

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IgG. These mixtures were then incubated for two hours on ice, in order to form tri-molecular conjugates b-catalase/SA/b-anti-ICAM or b-catalase/SA/b-IgG. The same procedure has been utilized to generate tri-molecular complexes b-streptokinase/SA/b-IgG, b-streptokinase/SA/b-anti-ICAM.

**Example 2: Interaction of radiolabeled antibodies with cultured human endothelial cells**

Cultivated cells (HUVEC) were cultured in gelatin-coated plastic dishes ("Falcon") using Medium 199 with Earle's salts supplemented with 10% fetal calf serum, 200 µg/ml endothelial growth factor from human brain and 100 µg/ml heparin, 2 mM glutamine, 100 mU/ml penicillin and 100 µg/ml streptomycin. Cells were subcultivated from first to third passage by treatment with 0.05% trypsin/0.02% EDTA mixture.

To determine the internalization of antibodies by the endothelium, cells were incubated with 300 µl of culture medium containing 1 µg <sup>125</sup>I-anti-ICAM for 90 minutes at 37 C. After washing to remove unbound radioactivity, cells were incubated with 50 mM glycine, 100 mM NaCl, pH 2.5 (15 minutes at room temperature) to release surface associated antibody. There was no detectable cell detachment after treatment with glycine buffer as determined by light microscopy. After collection of the glycine eluates, cells were detached by incubation with standard trypsin/EDTA solution. Surface associated radioactivity (i.e., radioactivity of the glycine eluates) and cell associated radioactivity (i.e., radioactivity of trypsin/EDTA extracts) were determined in a gamma counter. Percent of internalization was calculated as

$$\% = (\text{total radioactivity} - \text{glycine eluted}) \times 100 / \text{total radioactivity}.$$

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**Example 3: Temperature dependence of pulmonary uptake of anti-ICAM-1**

Sprague-Dawley male rats, weighing 170-200 grams, were anesthetized with sodium pentobarbital, 50 mg/kg, i.p., and prepared for isolated lung perfusion using recirculating perfusate. The trachea was cannulated and lungs were ventilated with a humidified gas mixture (Airco Inc., Philadelphia, PA) containing 5% CO<sub>2</sub> and 95% air. Ventilation was performed using a SAR-830 rodent ventilator (CWE Inc., Ardmore, PA) at 60 cycles/minute, 2 ml tidal volume, and 2 cm H<sub>2</sub>O end-expiratory pressure. The thorax was then opened and a cannula was placed in the main pulmonary artery through the transected heart. The lungs were isolated from the thorax and initially perfused in a non-recirculating manner for a 5 minute equilibration period, in order to eliminate blood from the pulmonary vascular bed. Then lungs were transferred to the water-jacketed perfusion chamber maintained at 37°C or 6°C. Perfusion through the pulmonary artery was maintained by a peristaltic pump at a constant flow rate of 10 ml/minutes. The perfusate (45 ml per lung) was Krebs-Ringer buffer (KRB, pH 7.4), containing 10 mM glucose and 3% fatty acid-free BSA (KRB-BSA). Perfusate was filtered through a 0.4 µm filter prior to perfusion to eliminate particulates. To quantitate antibody binding, 1 µg of <sup>125</sup>I-labeled anti-ICAM-1 antibody 1A29 was added to the perfusate. Perfusate circulated for 60 minutes at either 37°C or 4°C. Then non-bound material was eliminated by 5 minutes non-recirculating perfusion of antibody-free KRB-BSA. Radioactivity in the lungs was measured in a gamma-counter and expressed as a percentage of perfused radioactivity per gram of lung tissue (% ID/g).

**Example 4: Rat lung perfusions with biotinylated mAb 1A29**

Perfusion of isolated rat lungs was performed as above. At the first step, 10 µg of non-labeled biotinylated anti-ICAM-1 antibody was added to the perfusate and circulated for



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30 minutes at 37°C, to allow for antibody binding with the pulmonary endothelium. Thereafter, non-bound antibody was eliminated as above and perfusate was replaced with antibody-free KRB-BSA. Lungs were further perfused for either 5 minutes (to minimize antibody internalization) or 60 minutes at 37°C (to allow for antibody internalization). At the indicated time, 1 µg of <sup>125</sup>I-labeled streptavidin was perfused for 15 minutes with recirculating perfusion at 37°C followed by 5 minutes with non-recirculating perfusion with KRB-BSA to eliminate non-bound material. Radioactivity in the lungs was measured in a gamma-counter and expressed as a percentage of perfused radioactivity per gram of lung tissue (% ID/g).

**Example 5: Pulmonary uptake of <sup>125</sup>I-tPA and <sup>125</sup>I-streptokinase conjugated with anti-ICAM-1 mAb 1A29 in perfused rat lungs**

To study pulmonary uptake of radiolabeled preparations in blood-free buffer, 0.5 ml of saline containing 1 µg of radiolabeled b-streptokinase or b-tPA conjugated with anti-ICAM-1 antibody 1A29 was added to the perfusate and circulated in the isolated rat lungs for 1 hour at 37°C, as described above. Control lungs were perfused with complexes containing b-IgG instead of b-anti-ICAM-1. After a one hour perfusion, non-bound material was eliminated and lung-associated radioactivity was determined as above.

**Example 6: Pulmonary uptake of <sup>125</sup>I-tPA and <sup>125</sup>I-streptokinase conjugated with anti-ICAM-1 mAb 1A29 after injection in vivo in rats**

To study biodistribution of radiolabeled preparations in rats, an injection of 0.5 ml of saline containing 1 µg of radiolabeled b-streptokinase or b-tPA conjugated with anti-ICAM-1 antibody 1A29 was made into the tail vein under anesthesia. Control animals were injected with complexes containing b-IgG instead of b-anti-ICAM-1. Animals were

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sacrificed by exsanguination 60 minutes after injection. Internal organs were washed with saline to remove blood and radioactivity in tissues was determined in a Rack-Gamma counter. The data were calculated as mean  $\pm$  standard error (M  $\pm$  SE). Statistical comparisons were made using one-way analysis of equal variance (ANOVA) followed by Student-Newman-Keuls Method. The level of statistical significance was taken as  $p < 0.05$ .

**Example 7: Functional activity of tPA in isolated rat lungs**

To characterize functional activity of tPA conjugated with anti-ICAM antibody, 0.5 ml of saline containing 1  $\mu$ g of b-tPA conjugated with anti-ICAM-1 antibody 1A29 was added to the perfusate and circulated in the isolated rat lungs for 1 hour at 37°C, as described above. Control lungs were perfused with complexes containing b-IgG instead of b-anti-ICAM-1. After a one hour perfusion, non-bound material was eliminated and lung homogenates were prepared. To test fibrinolytic activity of the homogenates, radiolabeled fibrin clot was prepared by addition of 50  $\mu$ l of thrombin solution (1  $\mu$ g/ml in saline) to a solution of radiolabeled human fibrinogen (3 mg/ml in KRB). Immediately after thrombin addition, aliquots of the solution (300  $\mu$ l) were made and allowed to polymerize (60 minutes at room temperature). This procedure provides standard fibrin clots containing radiolabeled fibrin. Saline (1 ml) containing 50  $\mu$ l of the homogenates prepared from lungs perfused with either b-tPA/SA/b-anti-ICAM or b-tPA/SA/b-IgG complexes (see above) was added to fibrin clots. After a 2 hour incubation at 37°C, radioactivity in the supernatants was determined. Percent of fibrinolysis was expressed as percent of the radioactivity in the supernatants (i.e., radioactivity of the products of fibrin degradation) to the total radioactivity of fibrin clots.

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What is Claimed is:

1. A method for targeting and prolonging association of a selected drug to the luminal surface of pulmonary vascular endothelium of an animal comprising administering to an animal  
5 a selected drug in combination with a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vascular endothelium.

2. The method of claim 1 wherein the non-internalizable antibody is an anti-ICAM-1 antibody.

10 3. The method of claim 1 wherein the selected drug is conjugated with the non-internalizable antibody.

4. The method of claim 1 wherein the non-internalizable antibody is administered to the animal followed by administration of the selected drug wherein the selected  
15 drug has been chemically modified to recognize the non-internalizable antibody associated with luminal surface of the pulmonary vasculature.

5. A method for dissolution of fibrin clots or prevention of the intravascular coagulation in the pulmonary  
20 vasculature of an animal comprising administering to the animal a fibrinolytic or anticoagulant in combination with a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vascular endothelium.

6. The method of claim 5 wherein the non-internalizable  
25 antibody is an anti-ICAM-1 antibody.

7. The method of claim 5 wherein the fibrinolytic or anticoagulant is conjugated with the non-internalizable antibody.

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8. The method of claim 5 wherein the non-internalizable antibody is administered to the animal followed by administration of the fibrinolytic or anticoagulant wherein the fibrinolytic or anticoagulant has been chemically modified  
5 to recognize the non-internalizable antibody associated with luminal surface of the pulmonary vasculature.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/17386

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 39/395

US CL :424/134.1, 178.1, 181.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/134.1, 178.1, 181.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PANES et al. Regional differences in constitutive and induced ICAM-1 expression in vivo. Amer. J. Physiol. 1995, Vol. 269, pages H1955-H1964, see entire document.	1-8
Y	RUNGE et al. Antibody-enhanced thrombolysis: Targeting of tissue plasminogen activator in vivo. Proc. Natl. Acad. Sci. USA. November 1987, Vol. 84, pages 7659-7662, see entire document.	1-8
Y	IMAIZUMI, T. Effect of Antibodies Against Neutrophil and Endothelial Adhesion Molecules on Reperfusion Injury After Pulmonary Ischemia. Transpl. Proc. August 1994, Vol. 26, No. 4, pages 1851-1854, see entire document.	1-8

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*g* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search      Date of mailing of the international search report

22 OCTOBER 1999

23 DEC 1999

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/17386

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MULLIGAN et al. Tumor Necrosis Factor $\alpha$ Regulates in Vivo Intrapulmonary Expression of ICAM-1. Amer. J. Pathol. June 1993, Vol. 142, No. 6, pages 1739-1749, see entire article.	1-8
Y	MUZYKANTOV et al. Immunotargeting of erythrocyte-bound streptokinase provides local lysis of a fibrin clot. Biochim. Biophys. Acta. 1986, Vol. 884, pages 355-362, see entire article.	1-8
Y	TORCHILIN et al. Long acting thrombolytic immobilized enzymes. J. Contr. Rel. 1985, Vol. 2, pages 321-330, see entire article.	1-8
Y	MUZYKANTOV et al. Endothelial cells internalize monoclonal antibody to angiotensin-converting enzyme. Amer. J. Physiol. 1996, Vol. 270, pages L704-713, see entire article.	1-8
Y	RUNGE et al. Conjugation to an Antifibrin Monoclonal Antibody Enhances the Fibrinolytic Potency of Tissue Plasminogen Activator in Vitro. Biochem. 1988, Vol. 27, pages 1153-1157, see entire article.	1-8
Y	BOWES et al. Monoclonal Antibody to the ICAM-1 Adhesion Site Reduces Neurological Damage in a Rabbit Cerebral Embolism Stroke Model. Exp. Neurology. 1993, Vol. 119, pages 215-219, especially page 216, column 1 and page 218, Summary.	1-8
Y	BOWES et al. Monoclonal antibodies preventing leukocyte activation reduce experimental neurologic injury and enhance efficacy of thrombolytic therapy. Neurology. April 1995, Vol. 45, pages 815-819, see entire article.	1-8

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/17386

## B. FIELDS SEARCHED

Documentation other than minimum documentation that are included in the fields searched:

STN (Biosis, Medline, Embase, Caplus), WEST

search terms: Murciano, Juan Carlos, Granger, D. Neil, ICAM, mab 1A29, tPA, streptokinase, anticoagulant, non-inemalizable, antibody, pulmonary, thrombolysis, clot, pulmonary reperfusion